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Immunology

Interferon-γ regulates an antigen specific for endothelial cells involved in lymphocyte traffic

(endothelium/homing/migration/inflammation)

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ABSTRACT **One of the most striking examples of localized vascular differentiation is exhibited by specialized lymphoid organ venules that mediate the extravasation of circulating lymphocytes from the blood. These vessels are characterized by cuboidal or "high" endothelial cell morphology and are unique in their functional capacity to interact with migrating lymphocytes, regulating both the rate and specificity of lymphocyte traffic through particular regions of the body. We describe here a monoclonal antibody, MECA-325, that defines an endothelial cell differentiation antigen selectively expressed on high endothelium in the mouse. Thus an antigen defining a specific functional subset of endothelial cells has been found. Furthermore, we demonstrate that the MECA-325 antigen can be induced in mouse lung or bone marrow-derived endothelial cell lines *in vitro* by interferon-γ but not by interferon-β, interleukin-1, or endothelial cell mitogens. The results define a unique marker associated with differentiated endothelial cells mediating lymphocyte traffic from the blood, and they provide evidence that the specialized phenotype of these high endothelial cells may be induced and controlled by local factors associated with immune activity.**

Circulating lymphocytes leave the blood by binding to the specialized endothelium of high endothelial venules (HEV), subsequently migrating across the vessel wall into the surrounding tissue (1). Studies in murine (2–6) and human (7) systems have shown that lymphocytes express specific receptors for organ-specific ligands on HEV in mucosal lymphoid organs and peripheral lymph nodes. Thus HEV can control the traffic of lymphocyte subsets through particular lymphoid tissues. HEV are also observed in sites of antigenic insult, where they are thought to mediate the increased lymphocyte traffic associated with chronic inflammation (8, 9, 24). The nature of HEV, and of the factors controlling their appearance in lymphoid tissues and in sites of inflammation, is thus of interest in the context of both the regulation of endothelial cell differentiation and the control of local immune responses.

Here we describe a monoclonal antibody, MECA-325, that defines a differentiation antigen selectively expressed by HEV in the mouse, and we demonstrate that this antigen is specifically induced in cultured lung and bone marrow endothelium by interferon (IFN)-γ. The results support the concept that endothelial cell specialization may be determined by local microenvironmental factors, rather than by selective population of particular vessels by distinct endothelial cell lineages. They also indicate that lymphocyte traffic may be regulated in part by the induction of high endothelial differentiation in venules exposed to factors resulting from local immune reactivity.

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MATERIALS AND METHODS

Antibodies. Monoclonal antibodies against mouse high endothelium were raised by immunizing rats with a stromal preparation of mouse lymph nodes in complete Freund's adjuvant. A boost in saline was given 1–2 weeks later and, subsequently, the rat's spleen cells were fused with Sp2/0 mouse myeloma cells (ATCC CRL 8006; American Type Culture Collection, Rockville, MD). Supernatants of hybridomas were screened on frozen lymph node sections by immunoperoxidase staining, and hybridomas producing monoclonal antibodies specific for endothelium were selected and cloned by limiting dilution. Two monoclonal antibodies defining mouse endothelial cell antigens, MECA-20 and MECA-325, were utilized in the current studies; they are of the IgG2a and IgG1 isotypes, respectively, as determined by Ouchterlony analysis.

Other antibodies employed include anti-angiotensin-converting enzyme (ACE 3.1.1; a generous gift of R. Auerbach) (10) and rat IgG2a Hermes-2, specific for a human lymphocyte surface antigen) and IgG1 [J4.B anti-NIP (NIP is 4-hydroxy-3-iodo-5-nitrophenylacetic acid); from R. Coffman, DNAX Research Institute, Palo Alto] negative control antibodies.

Immunohistologic Staining. A two-step immunolabeling technique was used; acetone-fixed frozen sections (6 μm thick) were incubated with the first antibody (hybridoma supernatant or purified antibody), washed, and incubated with a solution containing the second antibody coupled to horseradish peroxidase (goat anti-rat IgG/HRP from DAKO) and 5% normal mouse serum; horseradish peroxidase was visualized by incubation in a diaminobenzidine/H_2O_2 solution.

Cell Cultures. LE-II and MCC endothelial cells and L-929 fibroblast cells were cultured on coverslips and incubated with the indicated agents 2–4 days (during which period the cells grew to confluency) in Dulbecco's modified Eagle's medium/10% fetal calf serum at 37°C in a 7% CO_2/93% air atmosphere. LE-II cells, a gift from A. Curtis (Glasgow, Scotland), were derived from lung microvasculature of AKR/J mice (11). Induction studies were carried out with cells in passages 15 to 30. MCC-cells were derived from C57BL mouse bone marrow and were a kind gift from D. Gospodarowicz (University of California, San Francisco). The cells were classified as endothelial cells by morphology; staining with endothelial cell-specific antibodies ACE 3.1.1 (10), MESA-1 (12), and MECA-20 (see text); expression of laminin and collagen type IV; and response to endothelial cell growth factors. In addition, LE-II cells have been reported to contain Weibel–Palade bodies (11) and to form tubular structures beyond confluency (13). The cells were grown on

Abbreviations: HEV, high endothelial venules; IL-1, interleukin 1; IFN, interferon; EGF, epidermal growth factor; FGF, fibroblast growth factor; ECGF, endothelial cell growth factor.

fibronectin-coated coverslips and used between passages 5 and 15. L-929 and NIH 3T3 murine fibroblasts were from the American Type Culture Collection. WEHI-3 myelomonocytic cells and the NFS-60 myeloid line were obtained from R. Coffman.

Interleukins and Growth Factors. Human interleukin 1 β (IL-1) was the purified protein from staphylococcal protein-A stimulated human peripheral blood lymphocytes (obtained from S. Gillis, Immunex Co., Seattle). It was used at 100 units/ml. Murine IFN-β was purchased from Lee Biomolecular and used at 100 units/ml. Recombinant murine IFN-γ was a gift from Genentech (South San Francisco) and used at 100 units/ml. Epidermal growth factor (EGF) from murine submaxillary glands was purified by HPLC as described by Nestor *et al.* (14). Fibroblast growth factor (FGF) purified by HPLC was a gift from D. Gospodarowicz. Endothelial cell growth factor (ECGF) purified from bovine brain by HPLC was a gift from T. Maciag (Revlon, Rockville, MD). EGF, FGF, and ECGF were used at concentrations (5–10 ng/ml) that were mitogenic for LE-II cells.

Immunofluorescence Staining. Immunofluorescence staining of cultured cells, grown on coverslips, was carried out at room temperature in medium without azide. First-stage antibodies were hybridoma supernatants or purified antibodies. For detection of surface antigen Ia, a monoclonal antibody (Becton Dickinson) against Ia.2 ($H2^k$ haplotype, expressed by LE-II cells and L929 cells) was used. After the first incubation step, the cultures were washed, and bound antibodies were detected with a fluorescein-conjugated second-stage antibody—rabbit anti-rat Ig (Sigma) for MECA-20 and MECA-325, or goat anti-mouse Ig (Pelfreeze) for ACE 3.1.1 and anti-Ia. After staining, cells were fixed with 3.7% paraformaldehyde in phosphate-buffered saline and examined by fluorescence microscopy. A patchy staining pattern was observed with all antibodies, reflecting redistribution of antigen cross-linked by first- and second-stage antibodies.

RESULTS

To generate markers defining unique differentiated features of high endothelial cells, we produced hybridomas from rat spleen cells immunized with stromal tissue from mouse lymph nodes, selecting monoclonal antibodies recognizing endothelium by immunohistologic staining of lymph node frozen sections. One monoclonal antibody, MECA-325, demonstrated remarkable specificity for HEV, failing in immunohistologic studies to stain any other vessels in lymph node sections (Fig. 1 *A* and *B*). MECA-325 also stained HEV in Peyer's patches, but it showed no reactivity with blood vessels in other tissues examined, including thymus, spleen, kidney, brain, and heart. HEV capable of supporting increased lymphocyte traffic are known to be induced in sites of long-standing inflammation as well. Therefore, we examined the expression of the MECA-325 antigen 2 weeks after local dermal injection of sheep erythrocytes in complete Freund's adjuvant. While vessels in control dermis were MECA-325-negative, MECA-325-positive venules, often lined by plump endothelial cells, were readily identified in association with mononuclear and lymphocytic infiltrates in the chronically inflamed tissues (Fig. 1 *C* and *D*). MECA-325 thus defines an endothelial cell differentiation antigen that is selectively expressed *in vivo* by the morphologically distinct high endothelial cells that mediate lymphocyte egress from the blood into lymphoid organs and that appears on presumptive HEV in sites of chronic inflammation. Immunofluorescence studies of high endothelial cells, in cell suspensions or cytocentrifuge preparations of enzyme-dispersed lymph node stromal cells, demonstrated that the MECA-325 antigen is expressed both at the cell surface and in the cytoplasm.

The specificity of MECA-325 for a functionally and morphologically differentiated subclass of endothelium makes it a unique probe to study the regulation of endothelial cell differentiation. To ask whether the differentiated features of HEV might be inducible in endothelium from any source, or whether HEV might instead represent a distinct lineage of endothelial cells, we attempted to induce high endothelial cell differentiation in cultures of endothelial cells from a non-HEV-containing source, using MECA-325 as a differentiation marker.

Cultured lines of normal mouse microvascular endothelium from lung (LE-II cells) were stimulated for 2–4 days with IL-1, IFN-β, IFN-γ, and a panel of endothelial cell growth factors, and expression of surface antigens was assayed by immunofluorescence (Table 1). Unstimulated LE-II cells did not stain with MECA-325, but were positive with monoclonal antibodies against more general markers of endothelial cells—MECA-20 [recognizing a cell surface determinant present on all mouse blood vascular endothelium (A.M.D., M. Kerkhove, R. Bargatze, and E.C.B., unpublished data)] and ACE 3.1.1 [against angiotensin-converting enzyme, an enzyme expressed by most endothelial cells (10)]. Treatment with IL-1, IFN-β, and the endothelial cell mitogens failed to induce the MECA-325 antigen. However, the endothelial cells stained brightly with MECA-325 after stimulation with IFN-γ. Similarly, induction of the MECA-325 antigen was observed after stimulation of bone marrow-derived endothelium (MCC cells) with IFN-γ. The antigen was first detectable after 2 days of stimulation, and it persisted thereafter. Essentially all cells in the stimulated cultures were positive (Fig. 2*A*). The absence of induction of MECA-325 by growth factors indicates that expression of the antigen is not linked simply to proliferative state.

Since MECA-325 is highly selective for high endothelial cells *in vivo*, it seemed likely that antigen expression in response to IFN-γ would be a specific property of endothelial cells *in vitro* as well. To test this, we examined the effect of IFN-γ on control fibroblast cultures. Since IFN-γ can induce major histocompatibility complex class II (Ia) molecules on a variety of cell types *in vitro*, including endothelial cells (15, 16), we assessed Ia expression as a positive control for the effect of interferon. When LE-II cells were incubated with IFN-γ, Ia and MECA-325 antigens were induced in parallel; both were consistently expressed on all endothelial cells from 3 days of stimulation (see Fig. 2). In contrast, L929 fibroblasts treated with IFN-γ expressed Ia but failed to express the MECA-325 antigen. This result demonstrates that the ability to express the MECA-325 determinant in response to IFN is not a general characteristic of cell lines *in vitro* and indicates that MECA-325 and Ia antigens are regulated by independent intrinsic mechanisms. Similarly, 4 days of incubation with IFN-γ also failed to induce detectable MECA-325 antigen on NIH 3T3 cells, NFS-60, a mouse myeloid line, or WEHI-3 myelomonocytic cells.

DISCUSSION

We have described a monoclonal antibody, MECA-325, that defines an endothelial cell differentiation antigen selectively expressed *in vivo* on high endothelial cells—the specialized endothelium of lymph node postcapillary venules that supports the exit of migrating lymphocytes from the blood. To our knowledge, this represents the first demonstration of an antigenic determinant defining a functionally unique subclass of endothelial cells in the mouse. The molecular nature of the antigenic determinant defined by MECA-325 and its possible role in lymphocyte–endothelial interactions in lymphocyte extravasation require further investigation. The ability to make monoclonal antibodies specific for differentiated sub-

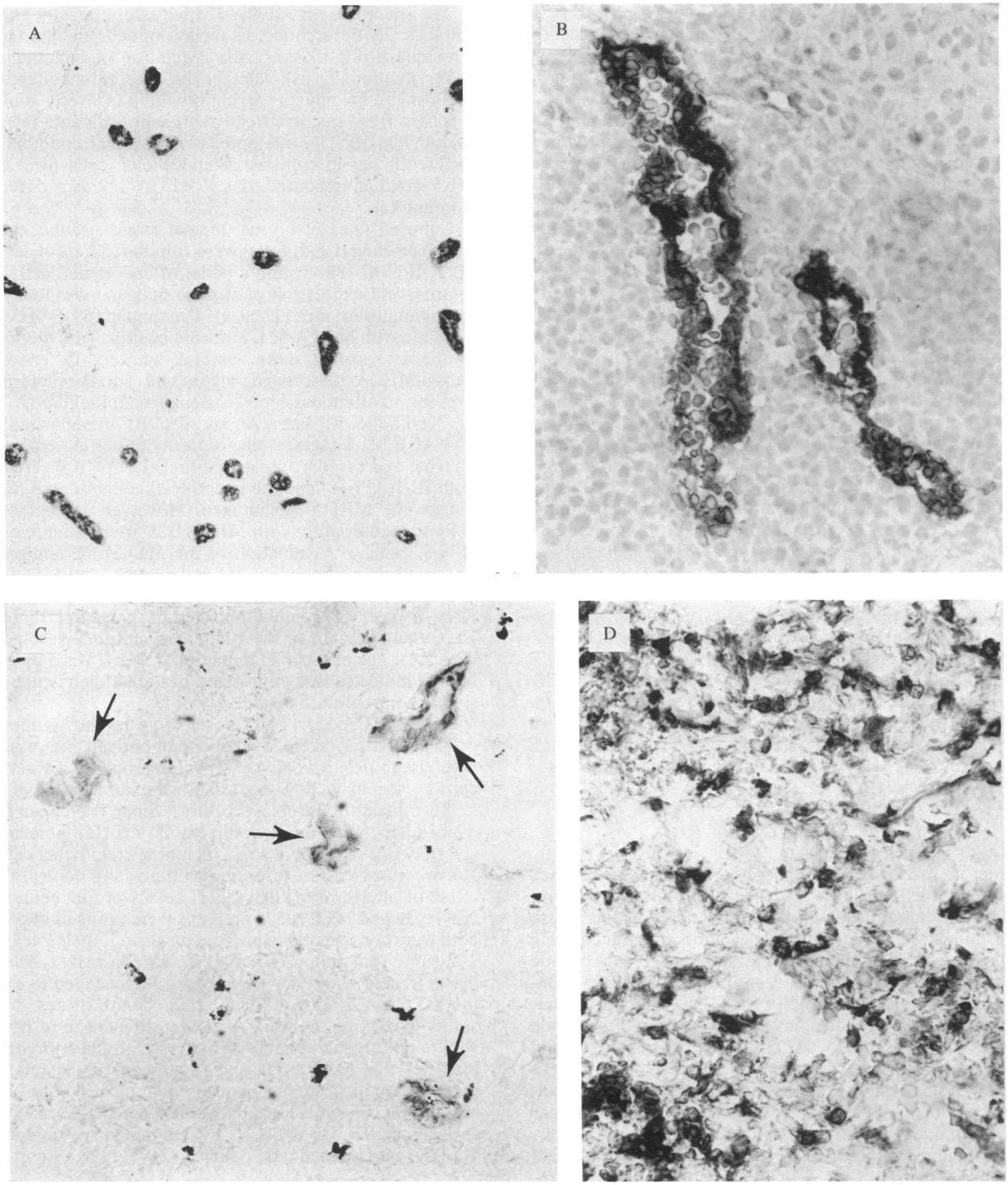

FIG. 1. Immunoperoxidase staining of mouse tissues with monoclonal antibody MECA-325. (*A*) MECA-325 selectively stains the endothelium of postcapillary HEV in mouse lymph nodes. (×100.) (*B*) Two HEV in a lymph node, darkly stained with MECA-325, with lymphocytes (lightly counterstained with hematoxylin) adhering to and migrating through the vessel wall. (×300.) (*C*) MECA-325-positive vessels with plump endothelial lining in a dermal site of chronic inflammation (2 weeks after local injection of sheep erythrocytes in complete Freund's adjuvant). (×250.) No reaction was seen in sections stained with J4.B, a class-matched negative control. (*D*) Serial section of the same inflamed site as in *C*, stained with anti-Thy-1, demonstrating the presence of numerous T lymphocytes in the infiltrate. (×250.)

sets of endothelium, using extremely crude tissue preparations as immunogen, may facilitate studies of endothelial cell differentiation *in vivo*; indeed, such approaches to defining functional states of endothelial differentiation may be essential, since cultured endothelial cells tend to dedifferentiate.

A central question in the biology of endothelium is whether morphologically and functionally distinct endothelial cell types represent separate lineages or can instead be induced from common endothelial cell precursors by local microenvironmental stimuli. We have shown that the MECA-325-defined high endothelial cell differentiation antigen is selectively induced in cultured endothelial cells from diverse sources by IFN-γ. Phenotypic differentiation of cultured endothelial cells has been reported in several other situations as well: Rat aorta segments in culture can be induced to capillary sprouting by fibrin clots (17, 18), and similarly,

Table 1. Induction of MECA-325 antigen in cultured endothelial cells by IFN-γ

Cell line	Antibodies	Staining of endothelial cells after treatment with: Untreated control	IFN-β	IFN-γ	IL-1	EGF	FGF	ECGF
LE-II	ACE 3.1.1	++	++	++	++	++	++	++
LE-II	MECA-20	+	+	+	+	+	+	+
LE-II	MECA-325	−	−	+++	−	−	−	−
LE-II	Control Abs	−	−	−	NT	NT	NT	NT
MCC	ACE 3.1.1	++	NT	NT				
MCC	MECA-20	+	NT	+				
MCC	MECA-325	−	NT	+++				
MCC	Control Abs	−	NT	−				
L-929	ACE 3.1.1	−	NT	NT				
L-929	MECA-20	−	NT	NT				
L-929	MECA-325	−	NT	−				

After 2–4 days of stimulation, control (nonstimulated) and stimulated cells were stained by immunofluorescence with the monoclonal antibodies ACE 3.1.1 (anti-angiotensin-converting enzyme), MECA-20, and MECA-325. Control monoclonal antibodies (Abs) included 1D1 (a rat IgG2a) and J4.B anti-NIP (a rat IgG1). All experiments were carried out two or more times. Level: negative (−) to intense (++++). NT, not tested.

cloned calf aortic endothelial cells can form capillary tubes *in vitro* (19). In addition, studies of quail–chicken transplantation chimeras demonstrated that abdominal vessels vascularizing grafted neural tissue form structural and functional features of a blood–brain barrier (20). Of particular relevance is the recent demonstration that IL-1 enhances the functional capacity of cultured human umbilical vein endothelium to bind neutrophils and myelomonocytic cell lines (21, 22). These observations, along with the data presented in this study, suggest that endothelial cells may be multipotential and can indeed differentiate according to influences from their local microenvironment.

Our results may also bear on the mechanisms controlling lymphocyte traffic into lymphoid tissues and into sites of inflammation *in vivo*. It is well known that morphologically characteristic HEV appear in response to chronic inflammation in extralymphoid tissues, where they are thought to support increased lymphocyte traffic into inflammatory sites (9, 24). In the studies reported here we have observed the appearance of MECA-325$^+$ venules in sites of subcutaneous Freund's adjuvant-induced chronic inflammation. Furthermore, Hendricks and Eesterman (23) have shown that the high endothelium in lymph nodes is also dependent on exogenous factors: they demonstrated that within 2–3 weeks after experimental interruption of the afferent lymph supply into rat lymph nodes, postcapillary venules lost their characteristic high endothelial morphology, and no longer supported lymphocyte extravasation from the blood. This loss of functional HEV was reversible by injection of antigen directly into the lymph node.

The current findings suggest that this regulation of HEV *in vivo* may be a direct response of endothelial cells to IFN-γ, and possibly other cytokines, generated during local immune reactivity. Thus the induction of high endothelial cell differ-

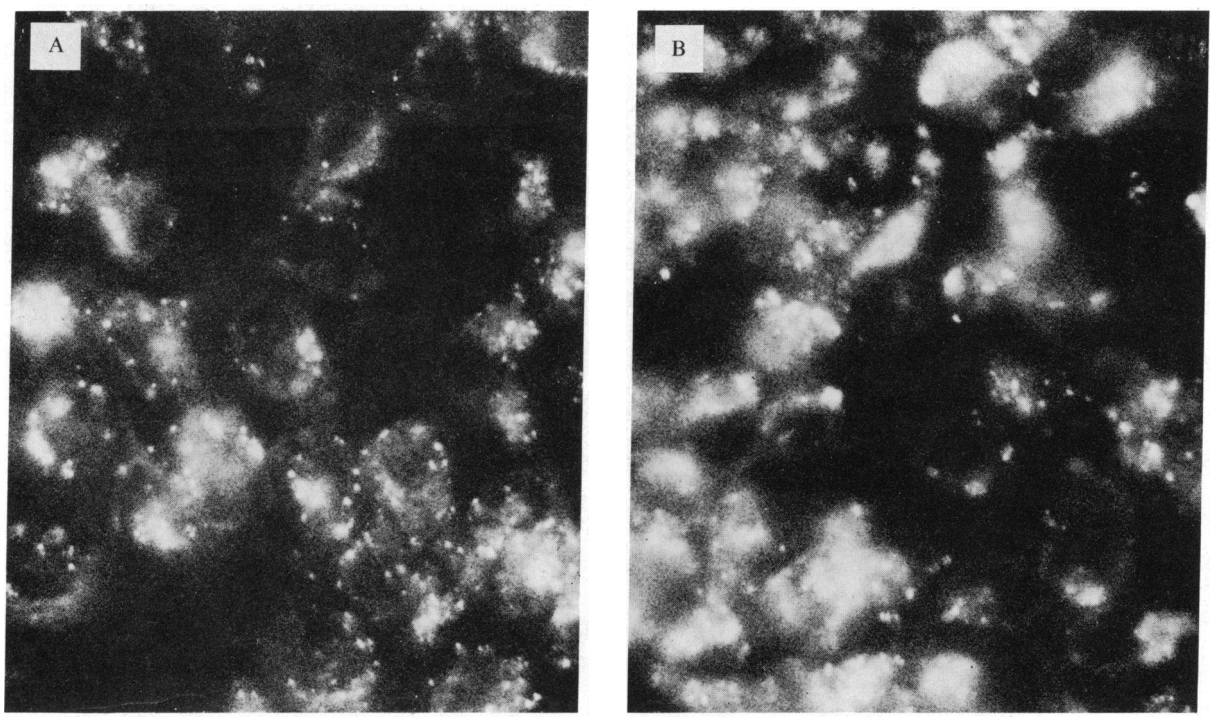

FIG. 2. Immunofluorescence staining of mouse LE-II endothelial cells after *in vitro* stimulation with IFN-γ. (×1000.) (*A*) MECA-325 staining, all cells are +++. (*B*) Anti-Ia.2 staining, all cells are ++++.

entiation, and of the capacity of endothelial cells to support lymphocyte traffic from the blood, may represent an important additional mechanism by which IFN-γ and other cytokines regulate local immune responses.

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